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Enhancement of Phloem Exudation from Cut Petioles by Chelating Agents[1]

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ABSTRACT

The photosynthetic assimilates in leaves of *Perilla crispa* attached to the plant were labeled by treating the leaves with $^{14}CO_2$. When subsequently detached, these leaves exuded a negligible amount of radioactivity from the cut petiole into water. Ethylenediaminetetraacetate (EDTA), citric acid, and ethyleneglycol-bis(β-aminoethyl ether) *N,N'*-tetraacetate greatly increased exudation of labeled assimilates into a solution bathing the petioles. The optimal concentration of EDTA was 20 mM, and maximal exudation took place between 2 and 4 hours after excision. Up to 22% of the radioactivity fixed in the leaf was exuded into an EDTA solution as compared to an export of 38% from attached leaves. The amount of radioactivity in the exudate was much reduced at low temperature. Presence of EDTA was required in the collecting solution for only 1 to 2 hours; upon transfer to water, exudation continued as in continuous presence of EDTA. Ca^{2+} completely inhibited the effect of EDTA.

Anatomical studies indicated that callose formation on the sieve plates near the cut surface of the petioles was less in leaves on EDTA than on water.

More than 95% of the radioactivity exuded by detached leaves was present in the sugars verbascose, stachyose, raffinose, and sucrose, which are translocated in the phloem of *Perilla*. Labeled glucose, fructose, and galactinol were detected in the leaf blade and petiole, but not in exudates.

The addition of EDTA to a solution bathing the petiole of detached leaves of *Chenopodium rubrum* and *Pharbitis nil* also increased the exudation of labeled assimilates. In these two species, label appeared only in a compound that cochromatographed with sucrose.

It is concluded that the radioactive products in the solution are actually exuded by the phloem. Possibly EDTA chelates Ca^{2+} that otherwise participates in the reactions that seal cut phloem.

Collection and analysis of phloem sap is possible by using the aphid stylet technique (16), or by allowing cut stems to exude (26). Such studies have established that, in addition to the transport sugars, phloem also contains amino acids, proteins, organic acids, various inorganic nutrients, and plant growth substances (6). Most species, however, will yield only a limited quantity of phloem sap from the end of a cut stem or petiole (18). This is presumably because, on cutting, the proteinaceous component of sieve tubes forms an aggregate, and callose is produced on the sieve plate pores (6). *In vitro*, reducing agents such as mercaptoethanol can prevent gelling of the proteinaceous material exuded from sieve tubes of *Cucurbita pepo* (24). Therefore, as a first step in attempting to collect relatively large quantities of phloem exudate, we have examined the effects of various compounds on exudation from the cut ends of petioles. The present communication establishes that EDTA stimulates exudation of labeled assimilates from detached leaves. The chemical nature of these assimilates is also described.

MATERIALS AND METHODS

Plant Material. Leaves were obtained from the short day plants *Chenopodium rubrum* L., selection 374 (origin 60° 47'N 137° 32'W), *Perilla crispa* (Thunb.) Tanaka, and *Pharbitis nil* Chois., strain Violet. The plants of *Perilla* were 2 to 3 months old and had been exposed to short days (8 hr light, 16 hr darkness) at about 23 C during the month prior to experimentation. The plants of both *Chenopodium* and *Pharbitis* were 4 to 6 weeks old and had been grown in continuous light from germination.

Photosynthesis with $^{14}CO_2$. Attached mature leaves of the plants were exposed to $^{14}CO_2$. In *Perilla*, leaves at the second or third node from the shoot tip were trimmed to an area of 30 cm^2 prior to feeding with $^{14}CO_2$. In *Chenopodium* the leaves were at node 9 or 10 and had attained an area of about 10 cm^2; in *Pharbitis* they were at node 3 or 4 and had attained an area of 50 cm^2. The leaves (generally 8 in an experiment) were enclosed in a Perspex assimilation chamber and fed $^{14}CO_2$ for 10 or 15 min under light from incandescent lamps (4000 ft-c filtered through a 5-cm layer of water). $^{14}CO_2$ was generated from 2 mg of $Ba^{14}CO_3$ (52 or 59 mc/mmole, unless otherwise stated) by addition of an aqueous solution of 20% lactic acid.

Exudation Techniques. Immediately after photosynthesis with $^{14}CO_2$ the leaves were cut off, and the petioles were recut under water. The leaves were placed in vials containing 5 ml of an aqueous solution at pH 7.0. Approximately 1.5 cm of the basal part of the petiole was immersed in the solution. Unless otherwise stated, the detached leaves were kept in darkness at 23 C.

The average amount of ^{14}C radioactivity (two to eight replicates in each treatment) that appeared in the solution bathing the petiole was taken as a measure of the effectiveness of various

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solutions and treatments in maintaining continued exudation from detached leaves. All exudation experiments were repeated at least once with similar results.

Aliquots of 0.1 ml of the exudation solutions were pipetted into vials, and radioactivity was counted in a liquid scintillation spectrometer, using a high counting efficiency formula capable of holding up to 4% water. This scintillation fluid consisted of: 100 g of naphthalene, 5 g of PPO, 0.3 g of dimethyl POPOP, 720 ml of dioxane, and 180 ml of toluene.

To relate the amount of labeled assimilate in the exudate to the amount of $^{14}CO_2$ fixed, radioactivity was determined following complete combustion of the sample in a Packard sample oxidizer. The released CO_2 was collected in ethanolamine, and radioactivity was counted in a liquid scintillation spectrometer.

A continuous measure of the translocation of ^{14}C from the fed leaves was obtained by monitoring radioactivity in the fed area (a 4-cm wide strip the width of the leaf) with a G-M detector (Nuclear-Chicago model 108). The detector was fixed in place under the fed area of the leaf blade, and its output from a ratemeter was recorded. Hofstra and Nelson (14) reported that this technique provides a good measure of the translocation of ^{14}C-assimilates.

Fractionation and Identification of Labeled Compounds. Labeled leaf blades and petioles were frozen in liquid nitrogen and lyophilized. The dry material was ground in a mortar with 80% ethanol and extracted 3 times at 70 C. The debris was centrifuged off, and the combined 80% ethanol supernatants were partitioned against petroleum ether (B.R. 60–80 C) to remove the lipid material. The 80% ethanol was evaporated to dryness and redissolved in 5 ml of water. The aqueous extracts were then passed successively through columns of Dowex 50 resin (1 × 5 cm; H^+ form), and Dowex 1 resin (1 × 5 cm; formate form). The neutral fraction containing the sugars passed through both columns. Amino acids were eluted from the Dowex 50 column with 4 N NH_4OH, and organic acids from the Dowex 1 column with 4 N formic acid. The effluents were evaporated to dryness and taken up in small volumes of 80% ethanol for counting of the radioactivity and for paper chromatography.

Aliquots of extracts and of exudates of *Perilla* plants were applied in small spots to sheets of Whatman No. 1 paper and first chromatographed in phenol-water (72:28, w/w) for 21 hr in one direction, and in *n*-butanol-propionic acid-water (10:5:7, v/v) for 15 hr in the second direction (4). Sugars were located by spraying with an ammonium molybdate reagent according to Aronoff (3, p. 98).

Radioactive compounds were located by exposing the chromatograms to Kodak no-screen x-ray films for 1 to 5 weeks, depending on the amount of radioactivity applied.

Sugars were identified by eluting each radioactive spot and rechromatographing the eluate along with standard sugars. As a final test, radioactive spots were eluted and hydrolyzed in 2 N trifluoroacetic acid at 105 C for 4 hr (1). The radioactive products obtained after hydrolysis were cochromatographed with glucose, fructose, and galactose, and the spots were located as before.

Exudates from leaves of *Chenopodium* and *Pharbitis* were lyophilized and taken up in a small volume of 80% ethanol, and the solutions were applied to strips of Whatman No. 1 chromatography paper which were 2 cm wide and 35 cm long. Descending runs of various durations established that no detectable radioactivity was eluted from strips run for 65 hr in the upper phase of a mixture of *n*-butanol-acetic acid-water (4:1:5, v/v) (25). At the same time ^{14}C-sucrose was run as a reference on a separate strip. Each strip was run through a chromatograph scanner to locate the position of radioactive materials.

Table I. *Effect of Various Compounds in the Collecting Solution on Exudation from Detached Perilla Leaves*

Four leaves per treatment; 6-hr exudation period in the light.

Collecting Solution	Radioactivity in Exudate
	cpm × 10^{-3}
Water	0
10 mM Mannitol	0
0.4 M Mannitol + 20 mM EDTA + 0.1% PVP + 50 mM 2-mercaptoethanol	560
10 mM Mannitol + 20 mM EDTA	900
20 mM EDTA	1,000

Callose Identification in Sieve Tubes. After detached *Perilla* leaves had been kept on water or EDTA for 6 hr, 2-mm segments were cut from the base of the petioles (five replicates per treatment). The specimens were fixed in 10% acrolein or 3% glutaraldehyde at 0 C, dehydrated, and then embedded in glycol methacrylate according to the methods of Feder and O'Brien (11). Transverse sections 3 μm thick, were cut 1.5 to 2.0 mm behind the basal end of the petiole. Next, longitudinal sections, also 3 μm thick, were cut. Following staining with a 0.1% solution of aniline blue (10), callose was localized by fluorescence microscopy. The callose-aniline blue complex fluoresces yellow when irradiated with UV at a wavelength of 365 nm. To assess callose formation quantitatively, the five replicates of each treatment were examined by the following four methods. (*a*) In transverse sections the number of sieve plates filled with at least 25% callose was determined for the total phloem cross sectional area. (*b*) In tangential sections using an eyepiece micrometer grid, the number of sieve plates filled with callose was counted per unit area of phloem in the region 0 to 600 μm behind the surface of the cut end of the petiole (average length of sieve tubes was 200 μm). (*c*) The thickness of 50 sieve plates filled with callose was measured in the same region as in method *b*. (*d*) The percentage of sieve plates filled with callose thicker than the sieve plate itself was also determined for the same region as in method *b*.

Chemicals. Disodium ethylenediaminetetraacetate dihydrate and citric acid were reagent grade (J. T. Baker Co.). Ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetate was obtained from Sigma Chemical Co. The chelating agents were dissolved in distilled water, and the pH was adjusted to 7.0 with 6 N KOH.

Polyvinylpyrrolidone (PVP), type NP-K60, with an average molecular weight of 160,000 was obtained from the GAF Corporation, New York.

RESULTS

Preliminary Experiments. In order to prevent gelling of the proteinaceous components (P-protein) present in the exudate from the phloem of *Cucurbita pepo*, Walker and Thaine (24) utilized a buffered solution containing 0.05 M 2-mercaptoethanol, 20 mM EDTA, 0.1% PVP,[3] and 0.4 M mannitol. When applied in an unbuffered solution (pH 7.0) to the base of cut petioles of *Perilla*, this same mixture of chemicals also caused exudation of labeled assimilates (Table I). No radioactivity

[3] Abbreviations: PVP: polyvinylpyrrolidone; EGTA: ethyleneglycol-bis(β-amino-ethyl ether) *N*,*N*′-tetraacetate.

Table II. *Effect of Temperature on Exudation from Detached Perilla Leaves*

Vials with the collecting solution were placed in ice and covered with several layers of aluminum foil. The petioles were inserted through small holes in the foil, so that the base of the petiole was at 0 C in the solution while the blade was kept at 23 C. Two leaves per treatment; 8-hr exudation period.

Collecting Solution	Temperature of Petiole	Radioactivity in Exudate
	C	cpm × 10^{-3}
Water	23	0
	0	0
EDTA	23	10,190
	0	850

leaked from petioles placed in water alone. When various components of the mixture were deleted, it was found that only EDTA was essential for continued exudation. The optimal concentration of EDTA for exudation over a 6-hr period was about 20 mM (2,050 × 10^3 cpm). Leaves exuded 30 × 10^3 cpm, 1,080 × 10^3 cpm, and 10 × 10^3 cpm into EDTA concentrations of 0.2, 2, and 200 mM, respectively. EDTA at a concentration of 20 mM has been used in all subsequent experiments.

Another condition favorable for exudation of ^{14}C label was the maintenance of the solution at a pH of at least 7.0. In one experiment, over a period of 8 hr, exudation into a solution of EDTA was 4,180 × 10^3 cpm at pH 4.4, 5,540 × 10^3 cpm at pH 5.8, and 8,590 × 10^3 cpm at pH 7.0. Exposure of leaves to 6 hr of intense light (3,500 ft-c) prior to feeding $^{14}CO_2$ reduced the amount of ^{14}C-label exuded by approximately 50% as compared to the amount of radioactivity obtained from leaves exposed to $^{14}CO_2$ immediately after their daily dark period (16,940 × 10^3 cpm *versus* 8,790 × 10^3 cpm). Low temperature also greatly reduced the amount of radioactivity exuded (Table II), indicating that active processes are involved in the transport or exudation of labeled products by detached leaves, or both.

In two other species, *Chenopodium rubrum* and *Pharbitis nil*, 20 mM EDTA also maintained exudation from detached leaves. Over a 6-hr period in darkness exudation was enhanced 28-fold in *Chenopodium*, but only by 40% in *Pharbitis*. Leaves of both these species exuded more radioactivity into water than did those of *Perilla*. Stimulation of exudation in *Pharbitis* by EDTA was only marginal. However, the leaves of this species wilted immediately when excised, and this wilting most probably prevented transport in the phloem (6).

Time Course of Exudation. In order to study the time course of exudation from detached leaves of *Perilla*, the labeled leaves were transferred at regular intervals after excision to vials containing fresh solutions of 20 mM EDTA. As illustrated in Figure 1, the rate of ^{14}C exudation reached a maximum between 2 and 4 hr after excising the leaves. Usually more than 80% of the radioactivity exported was obtained in the collecting solutions during the first 6 hr after excision. The decreased exudation of ^{14}C label in light as compared to darkness most probably reflects differences in the available pools of labeled and unlabeled assimilates. It can be derived from the data in Figure 1 that the time required for exudation of half the radioactivity ($t_{\frac{1}{2}}$) was approximately 2 hr both in light and in darkness.

In view of the preceding results, in subsequent experiments photosynthesis with $^{14}CO_2$ always took place at the end of the daily dark period, and during exudation the leaves were placed in darkness at 23 C.

Exudation from Detached Leaves and Export from Attached Leaves. Using a G-M detector, export of ^{14}C-labeled assimilates was monitored continuously from attached *Perilla* leaves held in light or darkness (Fig. 2). Although the timing of export was comparable to that of other species (15), leaves of *Perilla* in light exported comparatively little of their labeled assimilates. The average percentage (four experiments) of assimilates exported over a 6-hr period was 28%. In darkness, loss of ^{14}C label from *Perilla* leaves was increased at least 2-fold as compared to export in light (Fig. 2), but it must be realized that in darkness about 25% of the ^{14}C label is lost through respiration (see below). Thus, the true export curve for leaves in darkness levels off at 45% rather than at 70%. The time to half-export ($t_{\frac{1}{2}}$ in Fig. 2) from attached leaves was less than 2 hr.

In order to examine the effect of detaching a leaf on export of assimilates, radioactivity in detached leaves was determined by combustion of the samples and subsequent counting of the

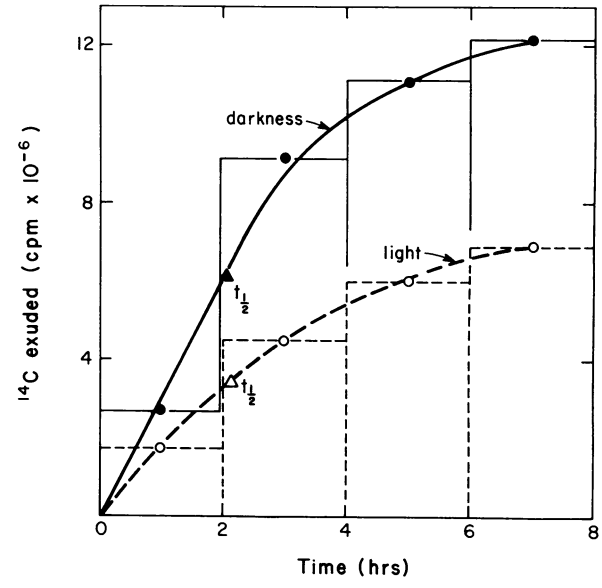

FIG. 1. Time course of exudation from detached *Perilla* leaves in light (fluorescent light, 200 ft-c) and in darkness. EDTA (0.02 M) present in the collecting solution. Eight leaves per treatment.

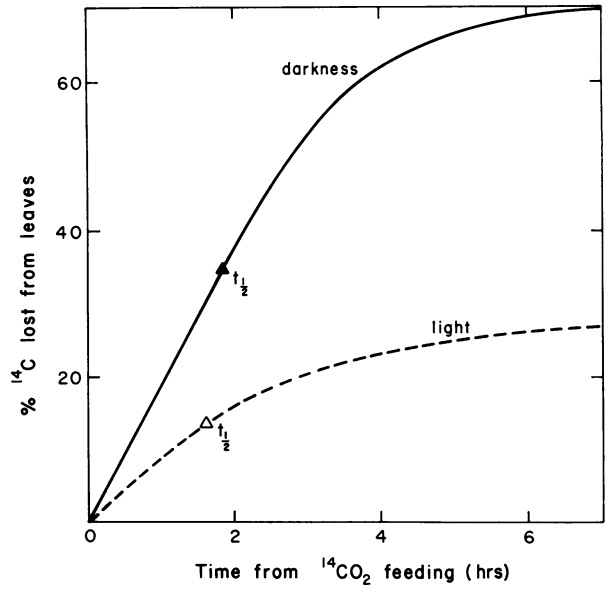

FIG. 2. Loss of ^{14}C from individual attached *Perilla* leaves. Dark treatment commenced immediately on cessation of a 3-min labeling period with $^{14}CO_2$. Light intensity 3500 ft-c.

CO_2 released. Detached *Perilla* leaves on water exuded no labeled assimilates and therefore lost 26% of their radioactivity through respiration (Table III). Comparable attached leaves lost 64% (36% remaining) of their radioactivity (compare 69% lost as monitored with G-M detector in Fig. 2). Thus, correcting for a 26% respiratory loss found in detached leaves, actual export of assimilates from attached leaves in darkness was 38% over a 6-hr period. Exudation over the same period amounted to 22% of the ^{14}C initially fixed by the leaves.

In further experiments with *Chenopodium* and *Pharbitis* combustion of the leaves was also used to assess the distribution of ^{14}C assimilates between detached leaves and the bathing solution without taking respiratory losses into account. It was found that after 6 hr in darkness *Chenopodium* and *Pharbitis* leaves had exuded into EDTA solutions 28 and 7%, respectively, of the final amount of radioactivity present in the leaf plus solution.

After-effect of EDTA. In the previous experiments EDTA was continuously present in the exudation solution. However, as shown in Table IV, experiment A, excised leaves will continue to exude if they are placed only for the first 1 or 2 hr on a solution of EDTA and then transferred to water. Presumably EDTA is taken up by the petiole and continues to exert its effect when the leaf is placed in water. This interpretation is supported by the following experiment. When a basal portion of 0, 1, 2, or 4 cm of the petiole was cut off after 1 hr in EDTA, and the leaves were then placed in water for 8 hours, the amount of radioactivity exuded declined as follows: $14,660 \times 10^3$ cpm, $6,040 \times 10^3$ cpm, $3,480 \times 10^3$ cpm, and 30×10^3 cpm, respectively. Apparently very little EDTA had moved over a distance of 4 cm or more from the base of the petiole.

Since removal of a slice of stem 1 mm in thickness causes exudation to resume in several species (6), in the experiment of Table IV, experiment A, a small piece of the petiole was also removed when leaves were transferred from water to EDTA. However, this was not essential for exudation to resume in *Perilla* (Table IV, experiment B). Leaves placed in water for a period of up to 8 hr and then transferred to EDTA without removing the basal portion of the petiole did exude a considerable amount of radioactivity. Thus, EDTA not only is able to prevent blockage of the cut petiole, but can also cause exudation to start up in previously plugged tissue.

Mechanism of Action of EDTA. The ability of EDTA to form stable complexes with divalent ions is well known, and the question arises whether this property of EDTA is also responsible for continued exudation from cut petioles. The following evidence indicates that this is indeed the case. The efficiency of EDTA as a chelating agent decreases with decreasing pH (5, 12), and the amount of radioactivity in the solution bathing the cut surface of the petiole of *Perilla* diminished also as the pH decreased from 7.0 to 4.4 (see above). Two other

Table III. *Comparison between the Amount of Radioactivity Exported from Attached Leaves and That Exuded by Detached Leaves*

Sixteen *Perilla* leaves were simultaneously labeled with $^{14}CO_2$, generated from 2 mg of $Ba^{14}CO_3$ (1.03 mc/mmole) for 10 min. During the 6-hr export or exudation period the leaves were kept in darkness. The total amount of ^{14}C per leaf with a 4-cm petiole was determined by combustion and counting of the CO_2 released. Immediately after exposure to $^{14}CO_2$ there were 408×10^3 cpm/leaf (=100%). Four leaves per treatment.

Treatment of Leaf	Radioactivity Remaining	Radioactivity Exported or Exuded
	%	%
Attached	36	38[1]
Detached, water	74	0
Detached, EDTA	51	22

[1] Corrected for a respiratory loss of 26%.

Table IV. *Effect on Exudation of Transferring Perilla Leaves from EDTA to Water, and from Water to EDTA*

Experiment A: At the time of transfer 2-mm slices were cut off at the base of the petiole. Two leaves per treatment. Experiment B: No piece of the petiole was cut off when leaves were transferred. Two leaves per treatment.

Collecting Solution	Radioactivity in Exudate	
	In first solution	In second solution
	cpm $\times 10^{-3}$	
Experiment A		
1 hr EDTA → 12 hr water	10	14,910
2 hr EDTA → 12 hr water	1,440	19,400
1 hr water → 12 hr EDTA	0	10,220
2 hr water → 12 hr EDTA	0	7,110
Experiment B		
24 hr EDTA		5,430
2 hr water → 22 hr EDTA	0	5,340
4 hr water → 20 hr EDTA	0	4,680
8 hr water → 16 hr EDTA	0	3,300

Table V. *Effects of Three Different Chelating Agents at pH 7.0 on Exudation from Perilla Leaves*

Two leaves per treatment; exudation period, 8-hr.

Collecting Solution	Radioactivity in Exudate
	cpm $\times 10^{-3}$
Water control	0
20 mM EDTA	4,480
20 mM EGTA	5,100
20 mM citrate	2,690

Table VI. *Effect of Ca^{2+} and Other Cations in the Presence of EDTA, or after EDTA Treatment on Exudation from Perilla Leaves*

Experiment A: 20 mM EDTA; pH of each solution adjusted to 6.5. Two leaves per treatment. 8-hr exudation period. Experiment B: Petioles in 20 mM EDTA for 1.5 hr, then in $CaCl_2$ for 12 hr. Two leaves per treatment.

Collecting Solution	Radioactivity in Exudate
	cpm $\times 10^{-3}$
Experiment A	
EDTA	8,540
EDTA + 30 mM KCl	10,870
EDTA + 30 mM $MgCl_2$	3,750
EDTA + 30 mM $CaCl_2$	0
Experiment B	
Water	11,000
10 mM $CaCl_2$	320
40 mM $CaCl_2$	110
80 mM $CaCl_2$	80

chelating agents, *viz.* EGTA and citric acid, also caused exudation from cut petioles (Table V).

EDTA binds divalent cations on an equimolar basis (12). Thus, if a particular salt is present in a concentration greater than that of EDTA, this will result in freely available ions. As shown in Table VI, experiment A, an excess of Ca^{2+} abolished exudation completely, suggesting that this cation participates in the reactions that seal cut phloem.

Transferring leaves from EDTA to $CaCl_2$ solutions strongly reduced the quantity of labeled assimilates exuded into the transfer solution as compared to the water control (Table VI, experiment B). In similar experiments several other divalent ions, *viz.* Mn^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+}, at a concentration of 10 mM, also reduced exudation, albeit somewhat less severely than Ca^{2+}. The stability constants of EDTA for these four cations are higher by 3 to 8 orders of magnitude than for Ca^{2+} (23). Consequently, any one of these cations will displace Ca^{2+} from its EDTA complex, and, apparently, as a result exudation is reduced.

Effect of EDTA on Callose Formation. Callose has often been implicated in the blockage of sieve tubes and, hence, of translocation. The amount of callose present in petioles was therefore examined after detached leaves had been kept on water, or on a solution of EDTA for 6 hr. Typical callose fluorescence was observed in tangential (Fig. 3, A, B) and in transverse sections (Fig. 3C), both in the presence (Fig. 3A) and absence (Fig. 3, B, C) of EDTA in the solution bathing the petiole. No bright fluorescence was noticeable in tissues other than the phloem, and only sieve plates and the lateral sieve areas in the side walls showed callose formation.

All four criteria used to determine the degree of callose formation indicated more callose in petioles on water than in those on an EDTA solution (Table VII). Many sieve plates showed callose fluorescence without the pores themselves being blocked (Fig. 3). Since tissue sections were not always properly oriented, it was often difficult to quantitate precisely the number of fluorescent sieve plates per unit area of phloem. Consequently, there was a large variability between replicates, and the differences in numbers of sieve plates filled with callose were not significant. Following treatment with EDTA the thickness of the callose deposited on the plates was less, and there were significantly fewer sieve tubes with callose in the lumen (Table VII).

Chemical Nature of the Labeled Products in Exudates, Leaves, and Petioles. After fractionation of the exudate from *Perilla* leaves into an acidic, basic, and neutral fraction more than 95% of the radioactivity was recovered in the neutral fraction. Analysis of exudates by two-dimensional paper chromatography and autoradiography showed that practically all radioactivity cochromatographed with the four sugars verbascose, stachyose, raffinose, and sucrose (Fig. 4A). These same four compounds also formed the bulk of labeled sugars present

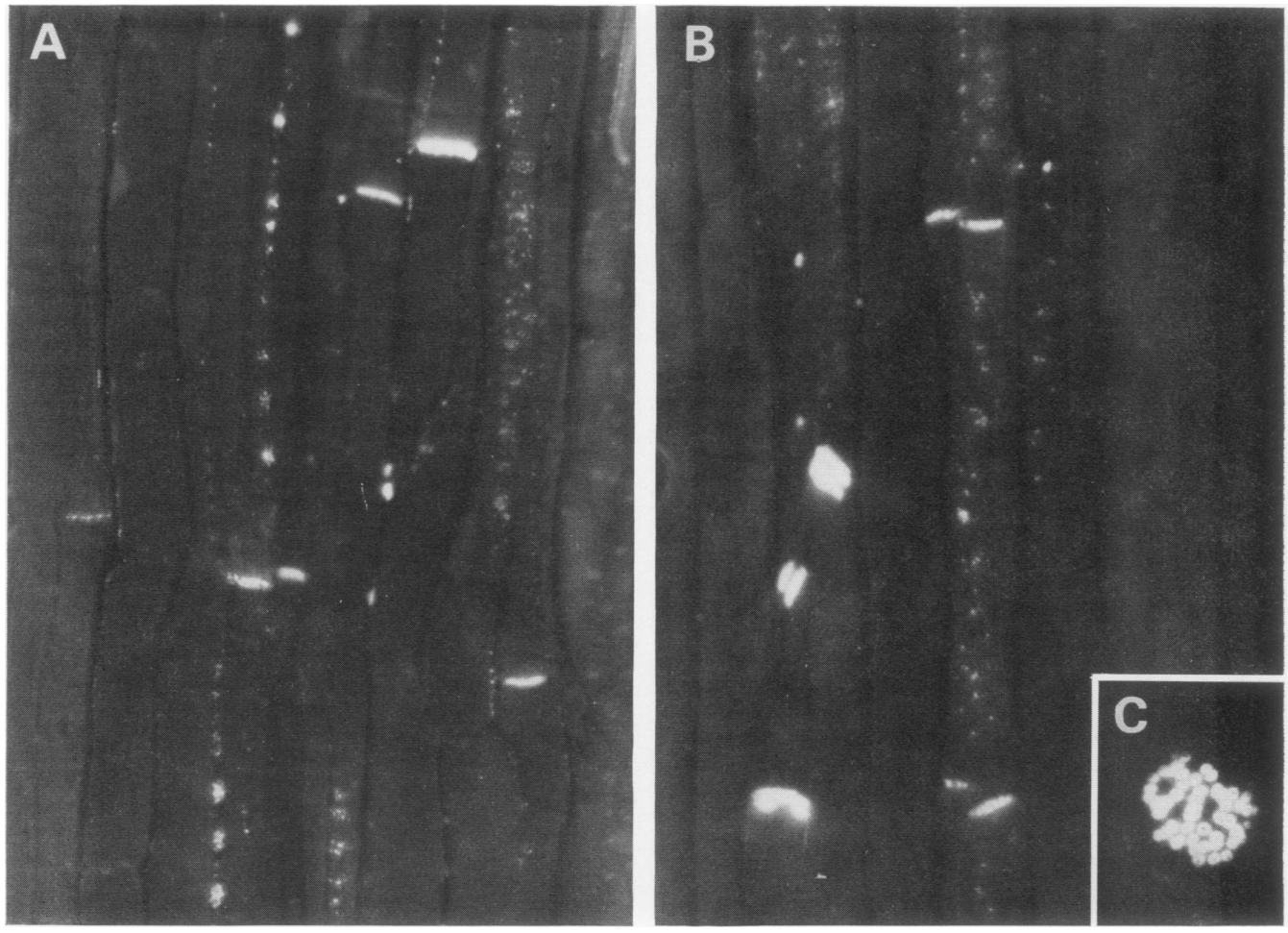

FIG. 3. A and B: Tangential sections at the base of the petiole of detached *Perilla* leaves on 20 mM EDTA (A), or water (B). × about 500. C: Cross section of the petiole of *Perilla* showing callose in the pores of sieve plates. × about 750.

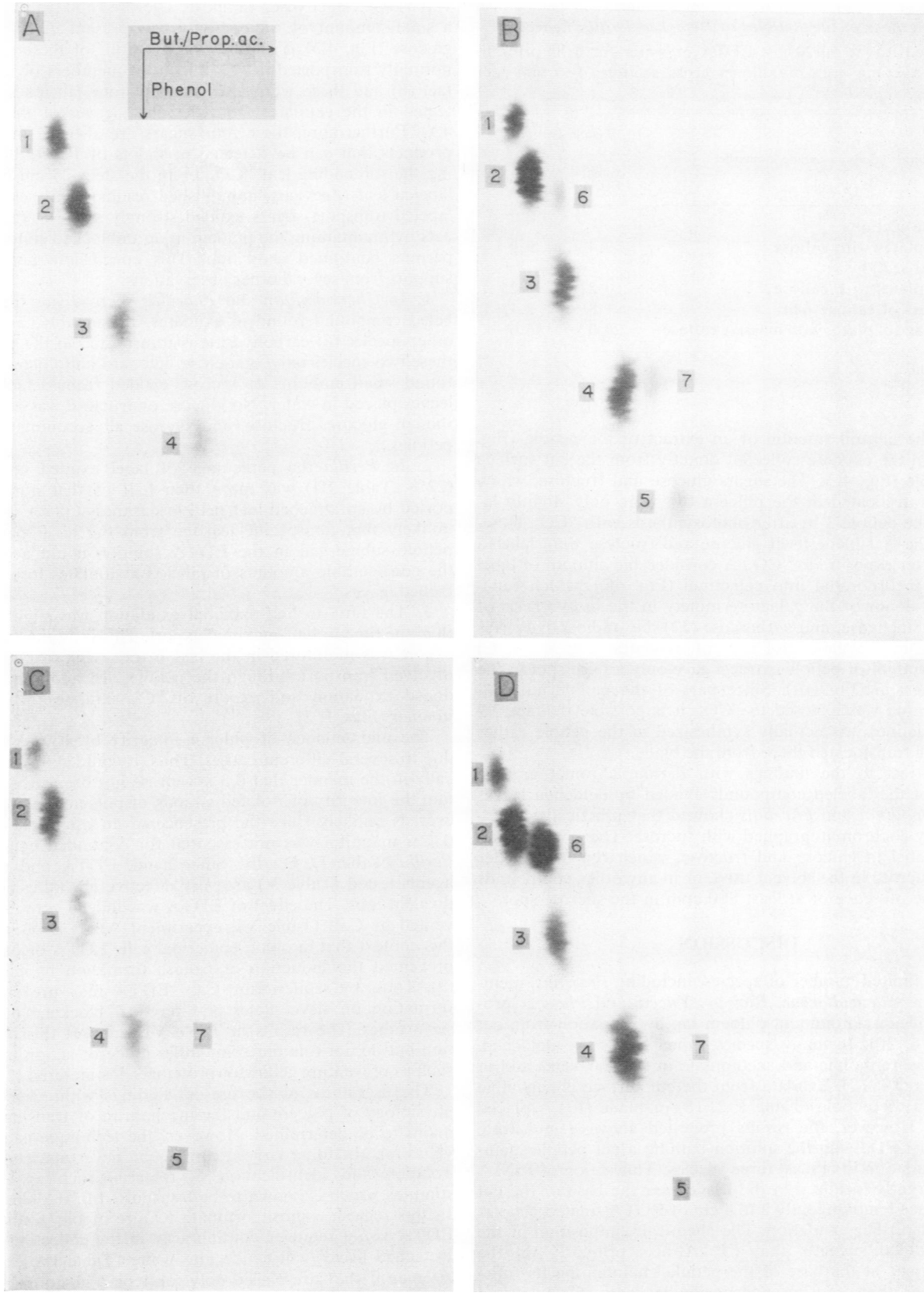

FIG. 4. Autoradiographs of two-dimensional paper chromatograms of products formed by *Perilla* after photosynthesis with $^{14}CO_2$. A: Exudate obtained from leaf with petiole in water for 11 hr after an initial period of 1 hr in EDTA. B: Neutral fraction of petiole extracted 3 hr after photosynthesis with $^{14}CO_2$. C: Exudate collected directly from the cut surface of a petiole 3 hr after photosynthesis with $^{14}CO_2$. D: Neutral fraction of leaf blade extracted after 15 min of photosynthesis with $^{14}CO_2$. 1: verbascose; 2: stachyose; 3: raffinose; 4: sucrose; 5: fructose; 6: galactinol; 7: glucose.

Table VII. *Effect of EDTA on Callose Formation in the Phloem at the Base of the Petioles of Detached Perilla Leaves*

Detached leaves on 20 mM EDTA or water for 6 hr prior to fixation. Average area of phloem cross section: 4.56 mm² per petiole.

Observations on Callose Formation	Petiole Treatment	
	20 mM EDTA	Water
Transverse sections		
No. of plates with callose	9.4	9.9
Tangential sections		
No. of plates with callose	0.22	0.25
Thickness of callose (μm)	1.81	2.12[1]
Percentage of plates with massive callose	26.0	46.0[2]

[1] $P < 0.05$.
[2] $P < 0.01$.

both in the neutral fraction of an extract of the petiole (Fig. 4B) and in the exudate collected directly from the cut surface of a petiole (Fig. 4C). The sugars glucose and fructose, which are not translocated in the phloem (6), were only slightly labeled in the petiole 3 hr after photosynthesis with $^{14}CO_2$. However, in the leaf blade itself glucose and fructose were labeled 15 min after exposure to $^{14}CO_2$. A considerable amount of label was also incorporated into galactinol (Fig. 4D), which functions as a donor of the galactose moiety in the biosynthesis of raffinose, stachyose, and verbascose (22). No radioactivity was detectable in the galactinol spot of exudates (Fig. 4, A, C) while galactinol of petiole extracts gave only a faint spot in the autoradiograph (Fig. 4B). Since part of the petiole adjacent to the lamina was exposed to $^{14}CO_2$, it is possible that the labeled galactinol was actually synthesized in the petiole rather than being translocated there from the blade.

In contrast to the findings with *Perilla*, chromatographic analysis of the labeled compounds exuded by detached leaves of *Chenopodium* and *Pharbitis* showed that practically all radioactivity cochromatographed with sucrose. There was no detectable label in glucose and fructose, which run at a higher R_F than sucrose in the solvent used, or in any other compounds despite the presence of at least 800 cpm in the sucrose spot.

DISCUSSION

Only a limited number of species, including *Fraxinus*, members of the *Cucurbitaceae*, *Ricinus*, *Yucca*, and *Phoenix* produce significant amounts of phloem sap by exudation from cut surfaces (6, 20). In many species, when leaves are detached, assimilates are mobile and accumulate in the leaf veins and in the petiole (18, 19). Exudate from the cut surface is negligible even when the petiole (Table I; 2, 18) or blade (13) is placed in water. However, the results presented above demonstrate that 20 mM EDTA in the solution bathing a cut petiole maintained exudation in at least three species. This action of EDTA was apparently localized in the tissue near the base of the cut petiole since removing only 2 to 4 cm of EDTA-treated petioles prevented further exudation. The chemical composition of the sugars exuded suggests that EDTA acts primarily on the phloem tissue at the base of the petiole, maintaining its function, rather than causing nonspecific leakage of all labeled products through increasing the permeability of all cells (8, p. 117). In *Perilla*, as in other members of the Labiatae (21) during photosynthesis in $^{14}CO_2$, label was readily incorporated into verbascose, stachyose, raffinose, sucrose, and galactinol, and a small amount of radioactivity was present in fructose and glucose (Fig. 4D). Yet, only the first four of these sugars are normally transported in the phloem of members of the Labiatae, (6) and these were also the only four labeled sugars detected in the exudates from detached leaves of *Perilla* (Fig. 4A). Furthermore, these four sugars are also the first labeled products that can be detected in shoots of *Perilla* after feeding the subtending leaf $^{14}CO_2$, or in the stem 25 cm below the labeled leaf (Zeevaart, unpublished results). This selectivity in labeled transport sugars exuded strongly suggests that EDTA acts by maintaining the phloem in an unblocked state and thus permits continued exudation. This conclusion gains further support from several other observations:

1. In *Chenopodium* and *Pharbitis* sucrose was the sole labeled compound found in exudates. Presumably, as in many other species (6) carbohydrate is transported in the phloem of these two species only as sucrose. Leonard and Glenn (18) also found small amounts of sucrose exuded from detached bean leaves placed in water. No glucose or fructose was exuded, although glucose, fructose, and sucrose all accumulated in the petioles.

2. In *Perilla* the percentage of label exuded in darkness (22%, Table III) was more than half of that normally exported by an attached leaf held in darkness (38%). Thus, it is unlikely that nonspecific leakage from the basal part of the petiole submerged in the EDTA solution could account for the considerable amounts of labeled assimilates lost from detached leaves.

3. The time to half-maximal exudation was slightly longer than the time to half-maximal export. This is as expected, since export was monitored in the leaf blades while exudation also involved transport through the petioles. In addition, light reduced exudation and export of ^{14}C-assimilates in a similar manner (Figs. 1, 2).

The maintenance of phloem integrity by EDTA could be due to several different causes. Thus, it could function osmotically in the manner that 0.3 M sucrose has been used to maintain the integrity of isolated strands of phloem (7). This, however, is unlikely since the presence of an osmoticum such as 0.4 M mannitol was not essential for continued exudation in *Perilla* (Table I). On the other hand, EDTA and the other agents tested (Table V) are very effective chelating agents for divalent ions. The effect of EDTA was indeed completely prevented by Ca^{2+} (Table VI, experiment A). It is of interest in this context that in onion epidermal cells EDTA or oxalic acid prevented the induction of callose formation by plasmolysis (9). Thus, by sequestering Ca^{2+}, EDTA may prevent callose formation on sieve plates and physical blockage of the cut sieve tubes. The results in Table VII support this interpretation but do not rule out some other mode of action of EDTA, such as preventing gelling of proteinaceous material (24).

The usefulness of the present findings with respect to the physiology of phloem and the mechanism of translocation remains to be determined. However, the technique is clearly of value for obtaining large quantities of sieve tube contents for isolation and identification of material such as the floral stimulus which is known to be transported in the phloem (17). In this regard, a most fortunate feature of our results is that EDTA is not required continuously in the collecting solution. Detached leaves will exude into water (Table IV, experiment A) even if they are placed only for 1 or 2 hr on a solution of EDTA.

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